Infections of cats with blood mycoplasmas in various contexts

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Abstract

Haemotropic microorganisms are the most common bacteria that infect erythrocytes and are associated with anaemia of varying severity. The aim of this study was to focus on the occurrence of *Mycoplasma haemofelis*, *Mycoplasma haemominutum*, and *Mycoplasma turicensis* in cats. We followed infected individuals' breeding conditions, age, sex, basic haematological indices, and co-infection with one of the feline retroviruses. A total of 73 cats were investigated. Haemoplasmas were detected by PCR and verified by sequencing. Haematology examination was performed focusing on the number of erythrocytes, haemoglobin concentrations and haematocrit. A subset of 40 cat blood samples was examined by a rapid immunochromatography test to detect retroviruses. The following was found in our study group: *M. haemofelis* in 12.3% of individuals, *M. haemominutum* in 35.6% of individuals and *M. turicensis* in 17.8% of individuals. A highly significant difference was found between positive evidence of blood mycoplasmas in cats living only at home (15%) and in cats with access to the outside (69.8%). There was also a highly significant difference in the incidence of mycoplasma in cats over 3 years of age compared to 1–3 years of age and up to 1 year of age. There was no difference in the frequency of infections between the sexes. Blood mycoplasma infection in our group was not accompanied by fundamental changes in the haematological indices and was only reflected by a decrease in haemoglobin values in three cases. In a subset of cats that were also examined for the presence of retroviral infection, mycoplasma infection in blood was confirmed in all five positive cases.

Blood pathogens, feline category, PCR, haematology

The haemotropic microorganisms found in cats include a group of bacteria that attack erythrocytes (Messick et al. 1998). They are transmitted by arthropods (Chomel et al. 2009). The three main bacterial pathogens tested include *Mycoplasma haemofelis*, *Mycoplasma haemominutum*, and *Mycoplasma turicensis*. Detection of these pathogens is associated with the development of molecular biological diagnostic methods due to the lack of reliable culture detection.

These mycoplasmas belong to the family *Mycoplasmataceae* and are referred to as agents of feline infectious anaemia (FIA). Based on the 16S rRNA gene sequence, two important species were identified in cats, *Mycoplasma haemofelis*, formerly *Haemobartonella felis* large (Hflg) and *Mycoplasma haemominutum*, formerly *Haemobartonella felis* small (Hfsm) (Niemark at al. 2001; Messick 2004). A new species of *Mycoplasma turicensis* was identified in 2005 (Willi at al. 2005). Their occurrence is global. *Mycoplasma haemofelis* causes severe macrocytic normochromic anaemia with the appearance of nuclear erythrocytes. *Mycoplasma haemominutum* causes no or mild haematological abnormalities (Foley et al. 1998). *Mycoplasma turicensis* may be the cause of haemolytic anaemia in cats (Santos et al. 2009). Blood mycoplasmas can be transmitted by infected blood (blood transfusions) or through lice, fleas, ticks and mosquitoes. Vertical transmission from mother to offspring is also possible. Transmission of these pathogens during fights

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is also likely. This theory is supported by studies showing the presence of haemoplasmic DNA in the saliva and claws of infected cats (Messick 2004). The incidence of blood pathogens of bacterial origin increases in immunosuppressed animals infected with Feline Immunodeficiency Virus (FIV) and Feline Leukaemia Virus (FeLV) (Tasker 2010). The risk group for the occurrence of FIV are uncastrated males older than 6 years (Sykes 2014). A FeLV infection may occur in very young animals. Despite the fact that blood mycoplasmas can cause serious clinical symptoms in their hosts or complicate the care of animals with retroviral infections, there is not yet enough information regarding their occurrence in the Czech Republic.

Taking into account these facts, this study focused on the general determination of the incidence of feline blood pathogens in animals from the Czech Republic that were subjected to veterinary examination, including a haematology analysis focusing on the erythrocyte count, haemoglobin level, and haematocrit. The aim of this study was to obtain information on the occurrence of these bacteria, to monitor their effect on haematological indices in individual cats as well as to draw attention to predisposing factors and focus on retroviral co-infection. The results of the study should increase the awareness of this group of pathogens among the veterinary profession, enrich the spectrum of common diagnostic methods and contribute to the targeted therapy of these infections.

Materials and Methods

Samples

We investigated blood samples from a total of 73 cats with different medical histories and different clinical manifestations. A total of 20 cats from the basic group (27%) came from a purely domestic environment (indoor), 53 cats (73%) had contact with the outdoor environment and were probably infested with ectoparasites. There were 23 females (31.5%) and 50 males (68.5%). The total population was divided into 3 age categories: 7 animals up to 1 year of age (10%), 12 animals aged 1–3 years (16%) and a total of 54 (74%) animals that were over 3 years old. The results of the basic haematological examination were available in the whole group of monitored cats. In this study, the following values were monitored: the erythrocyte count, the amount of haemoglobin and haematocrit. Blood was collected from the cats by veterinarians in a sterile way into test tubes with anticoagulant (EDTA) during basic veterinary examinations. After the haematological blood testing at the Central Clinical Laboratory, VetUni Brno (Celltac alpha MEK), blood was frozen at -20 °C.

Positive control strains

Positive control strains of mycoplasma were obtained from Dr. Messick of the Department of Comparative Pathobiology, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana, USA and were designed by Jensen at al. (2001).

Primers

For the amplification of *M. haemofelis* and *M. haemominutum*, the same primers were used to distinguish both pathogens based on the molecular weight of the resulting PCR products. For detection of *M. turicensis*, primers from Brazil were used (Santos et al. 2009). Primer sequences are shown in Table 1.

Table 1. Primer sequences.

Primer name	Primer sequence	Size/bp (base pair)	Reference
M. haemofelis for	5´ACG AAA GTC TGA TGG AGC AAT A-3´	170	Jensen et al. 2001
M. haemofelis rev	5´ACG CCC AAT AAA TCC GRA-3´		
M. haemominutum for	5´ACG AAA GTC TGA TGG AGC AAT A-3´	193	Jensen et al. 2001
M. haemominutum rev	5´ACG CCC AAT AAA TCC GRA-3´		
M. turicensis for	5´-GAA AAA TTT GAT GGT ACC CTC-3´	488	Santos at al. 2009
M. turicensis rev	5 ,-GCC GAA ACA CAA ATC CCG AC-3’		

Detection of *M. haemofelis, M. haemominutum*, and *M. turicensis* using PCR

Nucleic acids extraction and PCR: Total DNA was extracted by commercial extraction kits according to the manufacturer's instructions (NucleoSpin Blood DNA, Macherey-Nagel, Düren, Germany). Detection of bacterial DNA was done using Combi PPP Master Mix (Top-Bio, Prague, Czech Republic) by PCR in a total amount of 20 µl per sample according to the manufacturer's instructions. Reaction conditions were as follows: 95 °C/2 min, 94 °C/45 s, 53 °C/30 s, 72 °C/30 s, 34 ×, and final extension 72 °C/5 min for *M. haemofelis.* 95 °C/2 min, 94 °C/45 s, 54 °C/45 s, 72 °C/45 s, 36 × and final extension 7 min for *M. haemominutum* and for *M. turicensis.* Amplified PCR products were visualized in 2% agarose gel.

Gel extraction: Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd, New Taipei City, Taiwan) was used according to the manufacturer's instructions to extract the amplified DNA from agarose gel. The DNA fragments obtained were stored at -80 °C.

DNA sequencing of 16S rRNA gene of *M. haemofelis*, *M. haemominutum* and *M. turicensis*

The part of the genome which encodes the 16S rRNA gene of feline haemotropic bacteria was sequenced in Macrogen (Amsterdam, Netherlands). DNA sequences were compared with DNA sequences of reference strains from the GenBank database. Sequence analysis was performed by Geneious V 10.2.3. software (Auckland, New Zealand).

Detection of feline retroviruses

The FeLV antigen and FIV antibodies were detected by rapid immunochromatographic test (SNAP FIV/FeLV Combo Test, IDEXX, Westbrook, Maine, USA) in a total of 40 samples of blood.

Statistical analysis

Frequencies of pathogens in infected cats in different living conditions (indoor/outdoor), sex categories, different age groups and the influence of mycoplasmas on selected blood indices (haematocrit, haemoglobin, erythrocyte count) were compared on the basis of chi-square analysis of r × c and 2 × 2 contingency tables (with Yates correction) (Zar 1999). In the case of frequencies lower than five, Fisher's exact test was used instead of chi-square test. A P value < 0.05 was considered significant.

Results

Occurrence of blood mycoplasmas

Of the total of 73 samples examined by PCR and verified by sequencing, some of the blood mycoplasmas or a combination of them were detected in a total of 40 animals (55%). Ten animals were infected with multiple species at one time (25% of all positive cases). The presence of *M. haemofelis* was demonstrated in 9 cases (22.5% of all positive cases), *M. heamominutum* in 26 cases (65% of all positive cases) and *M. turicensis* in 13 cases (32.5% of all positive cases) (Table 2).

Table 2. Incidence of mycoplasma species.

Type of haemoplasma	Number of positive animals from a total of 73	Percentage of proof of species from all 40 positive cases
M. heamofelis	9 (12.3%)	22.5%
M. heamominutum	26 (35.6%)	65%
M. turicensis	13 (17.8%)	32.5%

Altogether, 20 cats from the basic group (27%) came from a purely domestic environment, 53 cats (73%) had contact with the outdoor environment (Table 3).

In cats kept at home, the presence of blood mycoplasmas was confirmed in 3 cases only (15%), of which two were co-infections with different types of haemoplasmas (Table 3). Cats with access to the outdoors were positive in 37 cases (69.8%) of which eight were co-infections with various types of haemoplasmas (Table 3). The difference in positivity rates between domestic and outdoor cats was determined to be highly significant ($P < 0.01$) in favour of cats in contact with the outdoor environment.

Table 3. Comparison of occurrence of blood mycoplasma between indoor and outdoor.

		Housing	
		Indoor	Outdoor
Total		20	53
Monoinfections	MHF	0	6
	MHM	1	17
	MT	0	6
Co-infections	MHF + MHM	1	1
	MHM + MT	1	6
	MHF + MT	0	1

MHF - *M. haemofelis*; MHM - *M. haemominutum*; MT - *M. turicensis*

Of a total of 73 cats, 23 (31.5%) were females and 50 (68.5%) males. Of the animals studied, 14 females (61%) were positive for the presence of mycoplasmas, of which three infections (21%) were mixed. Of the 50 males, the presence of haemoplasmas was demonstrated in 25 cases (50%), of which seven infections (14%) were co-infections (Table 4). There was no significant difference in the frequency of mycoplasma ($P > 0.05$) between females and males.

A total of 73 cats were further divided into 3 age categories (Table 5):

Table 4. Comparison of mycoplasmas between sexes.

		Female	Male
Total		23	50
Monoinfections	MHF	0	6
	MHM	7	10
	MT	4	2
Co-infections	MHF + MHM	1	1
	MHM + MT	2	5
	MHF + MT	0	1

MHF - *M. haemofelis*; MHM - *M. haemominutum*; MT - *M. turicensis*

Table 5. Comparison of mycoplasma incidence in terms of age.

		Up to 1 year	1–3 years	over 3 years
Total		7	12	54
Monoinfections	MHF	0	2	5
	MHM	0	2	15
	MT	3	1	3
Co-infections	MHF + MHM	0	1	1
	MHM + MT	1	3	3
	MHF + MT	0	0	1

MHF - *M. haemofelis*; MHM - *M. haemominutum*; MT - *M. turicensis*

In the youngest category up to 1 year, 4 animals were positive (57%), of which 1 was a mixed infection (25%). There were 9 (75%) positive animals in the 1–3 years category, of which 4 were positive for more than one haemoplasma (44%). In the category older than 3 years, positivity was found to be 52% (28 individuals); in five cases it was a co-infection by several species (18%).

For small numbers of individuals in the category up to 1 year and in the category 1–3 years, the results could not be objectively evaluated statistically.

Influence of mycoplasmas on selected blood indices

The erythrocyte, haemoglobin and haematocrit values were assessed for changes that might indicate anaemia. Tables 6, 7, and 8 show samples with haematocrit at the lower limit of the reference values. The reference ranges of these haematological indices are the following: haematocrit: 0.22–0.38 l/l; haemoglobin: 79–148 g/l; erythrocyte count: 4.9–9.8 × 10^{12}/l (Doubek et al. 2014). We focused on haematocrit values of 0.3 l/l and lower.

Table 6. Blood counts in *M. haemofelis* positive cats.

Environment, sex, age, disease	Erythrocytes (10^{12}/l)	Haemoglobin (g/l)	Haematocrit (l/l)
Outdoor, male, 5 years, periodontitis	7.59	101	0.3
Outdoor, male, 4 years, chronic hepatitis	5.8	77	0.23
Outdoor, female, 1.5 years, polytrauma	8.17	104	0.3
Outdoor, male, 10 years, apathy	6.68	93	0.26
Reference values	4.9-9.8	79-148	0.22-0.38

Table 7. Blood count values in *M. haemominutum* positive cats.

Environment, sex, age, disease	Erythrocytes (10^{12}/l)	Haemoglobin (g/l)	Haematocrit (l/l)
Outdoor, female, 10 years, trauma	7.25	90	0.3
Outdoor, male, 2 years, polytrauma, “no. 25”	4.73	75	0.22
Outdoor, male, 7 years, weight loss	6.64	97	0.3
Outdoor, female, 5 years, weight loss	8.13	100	0.3
Outdoor, female, 15 years, chronic kidney disease	6.96	93	0.28
Outdoor, female, 1.5 years, polytrauma	8.17	104	0.3
Indoor, male, 12 years, septic inflammation	6.08	95	0.3
Reference values	4.9-9.8	79-148	0.22-0.38

Table 8. Blood count values in *M. turicensis* positive cats.

Environment, sex, age, disease	Erythrocytes (10^{12}/l)	Haemoglobin (g/l)	Haematocrit (l/l)
Outdoor, female, 3 years, skin lesions, gingivitis	5.94	62	0.19
Outdoor, male, 2 years, polytrauma, “no. 25”	4.73	75	0.22
Outdoor, female,1 year, trauma	6.93	85	0.25
Outdoor, male, 7 years, apathy	6.18	93	0.29
Outdoor, male, 7 years, weight loss	6.64	97	0.3
Outdoor, female, 5 years, weight loss	8.13	100	0.3
Outdoor, male, 5 years, periodontitis	7.59	101	0.3
Reference values	4.9-9.8	79-148	0.22-0.38

Of the total of 9 samples positive for *M. heamofelis*, 4 animals (44%) had reduced haematocrit levels. Of the 26 samples positive for the presence of *M. heamominutum*, 7 animals (23%) had reduced haematocrit levels. Of the total of 13 samples positive for *M. turicensis*, 7 animals (44%) had reduced haematocrit levels. The differences in haematocrit values of *M. heamofelis, M. heamominutum* and *M. turicensis* were not significant ($P > 0.05$).

There was a non-significant difference between the individual blood counts in the three types of blood mycoplasma ($P > 0.05$). In three samples, lower haemoglobin values were found compared to the reference values. Sample no. 25 (male, 2 years old, outdoor, polytrauma) infected with *M. heamominutum* + *M. turicensis* showed a decrease in all three blood indices.

Examination for the presence of FeLV and FIV

A total of 40 cat blood samples that were previously screened for blood mycoplasmas by PCR were screened in the laboratory using SNAP test. The test demonstrates the presence of antibodies against FIV and the FeLV antigen in the blood of cats.

Of all 40 analysed samples, 5 cats infected with retroviruses (13%) were identified. The FeLV antigen was detected in 2 animals, antibodies against the FIV virus were detected in 2 animals and one animal was co-infected with both viruses (it was a chronically ill 8-year-old cat in which *M. haemofelis* was also detected). All five positive samples were also positive for blood mycoplasmas. Two samples showed co-infection of FeLV and *M. haemominutum*, two samples were positive for FIV and *M. haemominutum* and *M. turicensis.* One patient was positive for both retroviruses and *M. turicensis* (Table 9).

Table 9. Co-infection of blood mycoplasmas with retroviruses.

Retroviruses/blood mycoplasmas	MHM + MT	MHM	MT
FeLV		2	
FIV	2		
FeLV + FIV			1

MHM - *M. haemominutum*; MT - *M. turicensis*

Discussion

In clinical practice, we regularly encounter cases of undiagnosed transient anaemia, inappetence or poor fitness. Methods of molecular biology are of utmost importance in the diagnosis of feline diseases caused by blood mycoplasmas. Cultivation of feline blood to diagnose blood mycoplasmas is not routinely performed, as described by Greene (2006). Microscopic analysis of bacteria adhering to erythrocytes is possible, but the finding is not completely reliable because of the presence of various artefacts. We focused on the occurrence of blood mycoplasmas in feline blood using PCR and sequencing of the 16S rRNA gene region.

The prevalence of blood mycoplasmas reported in our monoinfection or co-infection study of 40 cats (55%) is much higher than in other European countries. This result may be due to different selections of cats and their total number in the research. Our study focused on the prevalence in cats, especially of outdoor type and higher age categories, with the predominance of cats over three years of age that had previously been infested with ectoparasites. Of the three mycoplasma species identified, *M. haemofelis*, *M. haemominutum*, and *M. turicensis* in the population of our cats, the prevalence of *M. haemominutum* in 26 animals was followed by *M. turicensis* in 13 animals, while the least frequently occurring mycoplasma was *M. heamofelis* in 9 animals. Blood mycoplasmas occur worldwide. A study of mycoplasma in cats in England gave the following results: 16.9% were positive for *M. haemominutum,* 1.4% were positive for *M. haemofelis* (Tasker et al. 2003). The following results were found in studies in Greece: 10.3% were positive for *M. haemominutum* and 7.2% were positive for *M. haemofelis.* No occurrence of *M. turicensis* in feline blood was observed (Maher et al. 2010). The overall incidence of blood mycoplasmas in cats in

southern Germany was only 9.4% in a similar study from 2016 (Bergmann et al. 2017). A Swiss study observed the prevalence of blood mycoplasma in a cat population using quantitative PCR in clinically healthy and diseased individuals. In contrast to our results, the authors reported a lower prevalence in both healthy and sick cats. *Mycoplasma haemominutum* was detected in 7% of healthy animals and in 8.7% of sick animals (anaemia), *M. haemofelis* in 2.3% and 0.2% of healthy and sick animals, respectively, and *M. turicensis* in 1.1% of sick cats only (Willi et al. 2006). In Italy, the prevalence of *M. haemofelis* was 4%, *M. haemominutum* 12.3%, both lower than in the Czech Republic. Half of the infected cats from the group were co-infected with several species of mycoplasma. The predisposing factor of feline infection was higher age and presence of FIV (Ravagnan et al. 2017). Cats infected with *M. haemominutum* usually display no clinical signs. On the other hand, *M. haemofelis* infection is usually manifested by anaemia and the resulting clinical symptoms.

There were a total of 20 indoor and 53 outdoor animals in our group of cats. Positive samples for mycoplasma came from 3 (15%) indoor cats and from 37 (69.8%) outdoor cats. Outdoor cats have a high probability of transmission due to fighting. Vectors such as fleas, lice and ticks are also known to be involved in the transmission of this infection. Three positive results in cats from indoor environment can be explained by the fact that owners may have reported the cat's current living conditions as indoor but the cat could have come into contact with the infection before it was under the care of the current owners. Another means of transmission may be if there is another animal (such as a dog, or another cat) living in the household together with the cat, that might have carried the vectors (fleas, ticks) transmitting infections indoors. A significant difference was found in the incidence of mycoplasma between indoor and outdoor environments. The most represented was *M. heamominutum* at the ratio of 1 indoor cat and 17 outdoor cats. These results are consistent with Danish experiments in which a significant difference was found between domestic outdoor cats and isolated domestic indoor cats (Rosenquist et al. 2016).

No significant differences were found in the incidence of mycoplasma in terms of sex. The mild differences between the sexes may be due to the aggressive behaviour of uncastrated males and their frequent rivalry and territorial fights, leading to the transmission of infections through scratches and bites. To reduce the number of positive cases, castration is recommended as it reduces aggressive behaviour towards other individuals. The biggest difference between the sexes was observed in *M. heamofelis* at a ratio of 8 positive reactions in males and 1 positive reaction in females. A study on the occurrence of mycoplasma in cats in southern Brazil has shown a highly significant difference between sexes at $P < 0.01$, with the predominant number of positive males (Santos et al. 2014). The outcome of the study is not consistent with ours, namely that blood mycoplasma infection occurs primarily in the male sex. Furthermore, an effect of age on the occurrence of blood mycoplasmas was observed. Most positive mycoplasma samples observed were in the category older than 3 years in 28 (52%) animals. A combination of several mycoplasmas in a single sample was observed in 10 individuals. Of these, 7 were simultaneously positive for *M. haemominutum* and *M. turicensis,* 2 were positive for *M. heamominutum* and *M. haemofelis,* and 1 was positive for *M. haemofelis* and *M. turicensis.* In the study from southern Brazil, a significant difference of $P < 0.05$ was demonstrated in mycoplasma occurrence with regard to age of. In the study, mycoplasmas were most prevalent in cats over 3 years of age, as was the case in our study (Santos et al. 2014).

In 40 mycoplasma-positive cats, the blood count obtained from the laboratory of the Department of Dog and Cat Diseases at VetUni Brno was also evaluated. Based on the haematological examination, the following categories were assessed: haematocrit value, haemoglobin level, and erythrocyte count in feline blood. Haemoplasmas causing various types of anaemia may be responsible for the decreased values of the aforementioned three

indices (Tasker at al. 2009). Blood count was monitored in 40 positive cats, of which 18 (45%) displayed reduced haematocrit levels.

The differences in haematocrit values in samples infected with *M. heamofelis, M. heamominutum* and *M. turicensis* were not significant. The difference in the erythrocyte count in *M. heamofelis* was not significantly different from *M. heamominutum* and *M. turicensis*. It was slightly decreased in one cat (no. 25). The mycoplasma species did not differ from each other in haemoglobin values either. These were reduced only in three cats, two infected with *M. turicensis,* and one infected with *M. haemofelis*. There are many adaptable mechanisms in a healthy animal, keeping the basic physiologic indicators at optimal levels in cats with quite a good health status (Doubek et al. 2014).

However, if haemotropic bacteria co-infect with other pathogens, they may potentiate the development of clinical symptoms. Cats infected with FIV or FeLV suffer much longer infections with blood mycoplasma due to immunosuppression (Tasker 2010). Of the two FeLV-positive cats, both were co-infected with *M. haemominutum* and one with FIV and *M. turicensis*. Two of the FIV positive cats were simultaneously infected with *M. haemominutum* and *M. turicensis* (Table 9).

In conclusion, this study summarizes observations and laboratory tests on blood mycoplasmas in cats. In the Czech Republic, laboratories only rarely perform blood mycoplasma tests using the PCR method, while blood smear microscopy is not sufficiently sensitive. This study offers a broader context for assessment and opens up the possibility of verifying clinical conclusions in the laboratory. Mycoplasmosis occurs at any age in cats, and up to a third of untreated animals die from severe anaemia (Sykes 2010). Therefore, it is important to pay particular attention to the occurrence of these pathogens and to focus especially on cats weakened by retroviral infections. The monitoring of animals divided in such manner was a "pilot study". From these unrepeatable results, consequences will be tracked down, and based on these results, exact groups of animals will be set up for another study.

The contribution of this study is in making available to the veterinary profession the diagnostic methods we have established, with optional testing of blood samples of suspect cats. Another future goal will be to introduce real time multiplex PCR (qPCR) to identify all major types of blood pathogens simultaneously. Our study has demonstrated a relatively high incidence of mycoplasmas in cat populations in the Czech Republic. Therefore, our focus going forward should be on the occurrence of these pathogens, especially in cats that show symptoms of anaemia, as well as on better evaluation of their impact on health, including the blood count.

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